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A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas

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Running Title: Meta-analysis of hESC transcriptome

Key words : pluripotent stem cells; gene expression profiling; microarray analysis

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Abstract

Microarray technology provides a unique opportunity to examine gene expression patterns in human embryonic stem cells (hESCs). We performed a meta-analysis of 38 original studies reporting on the transcriptome of hESCs. We determined that 1076 genes were found overexpressed in hESCs by at least 3 studies when compared to differentiated cell types, thus composing a “consensus hESC gene list”. Only one gene was reported by all studies: the homeodomain transcription factor *POU5F1/OCT3/4*. The list comprised other genes critical for pluripotency such as the transcription factors *NANOG* and *SOX2*, and the growth factors *TDGF1/CRIPTO* and *Galanin*. We show that *CD24* and *SEMA6A*, two cell surface protein-coding genes from the top of the consensus hESC gene list, display a strong and specific membrane protein expression on hESCs. Moreover, CD24 labeling permits to purify by flow cytometry hESCs co-cultured on human fibroblasts. The “consensus hESC gene list” also included the *FZD7* WNT receptor, the G protein-coupled receptor *GPR19*, and the *HELLS* helicase which could play an important role in hESCs biology. Conversely, we identified 783 genes downregulated in hESCs and reported in at least three studies. This “consensus differentiation gene list” included the *IL6ST/GPI30* LIF receptor. We created an online hESC expression atlas, (<http://amazonia.montp.inserm.fr>), to provide an easy access to this public transcriptome dataset. Expression histograms comparing hESC to a broad collection of fetal and adult tissues can be retrieved with this web tool for more than 15 000 genes.

Introduction

In the preimplantation mammalian embryo, the inner cell mass is able to differentiate into any cell type of the embryo proper. It has been recognized in mice since 1981 that embryonic stem cells (ESCs) with a prolonged proliferative capacity *in vitro* can be derived from the inner cell mass [1]. ESC line derivation from human embryos was reported in 1998 [2]. ESCs are pluripotent cells that can contribute to all tissues *in vivo*, and to the three primary germ layers as well as extraembryonic tissues *in vitro*. As pluripotency is maintained even after prolonged periods of culture, human ESCs (hESCs) have a great therapeutic potential in regenerative medicine. Careful molecular characterization of this unique cellular model of pluripotency should help to optimize and scale up *in vitro* manipulation of hESCs for clinical applications. Some genes specific to the very early stages of development and expressed in hESC lines such as *POU5F1/OCT3/4*, *NANOG*, *REX1*, *SOX2*, *FGF4* and *FOXD3* have already been identified [3-8]. However, the picture is far from complete and the molecular mechanisms involved in self-renewal and pluripotency are still under tight scrutiny [9, 10]. Moreover, extensive knowledge on hESCs should help in designing protocols for the isolation and production of other multi/pluripotent stem cells derived from tissues of adult individuals.

Microarrays are a major technical breakthrough that can monitor the expression of a whole genome in one experiment. Application of this technology to hESCs has largely contributed to our knowledge on the mechanisms underlying the maintenance of pluripotency of hESCs and their *in vitro* differentiation. Unfortunately, the datasets generated are heterogeneous both in accessibility (public databases or Supplemental data) and in the techniques used (variability in microarray design, sample labeling techniques, choice of control samples and computational tools). Despite our findings of 38 original publications reporting hESC transcriptome analyses, the disparities between datasets, the variety of

sources, the substantial know-how needed for transcriptome data mining discourage most non-specialists to consult this information. Thus, large amount of data are very much underused, and lack alternative interpretations since only the conclusions reflecting the analysis carried out by the authors are presented. This situation has led to initiatives such as *ONCOMINE* in the field of cancer [11]. We present here the first effort in compiling all publicly available microarray data relating to hESCs. From the 38 original publications studying the hESC transcriptome, we identified genes that were consistently overexpressed in hESCs when compared to differentiated samples (“consensus hESC gene list”) and underexpressed in hESCs (“consensus differentiation gene list”) in different studies. These lists will further deepen our knowledge on this unique cell model of developmental biology. Concurrently, we created an on-line database, *Amazonia!*, which provides an easy access to this public transcriptome dataset.

Materials and Methods

Lists of genes differentially expressed

Analyzing 38 original studies using transcriptome analysis to study hESC, we were able to collect 20 lists of transcripts that were upregulated in hESCs compared to differentiated cell types and 11 lists of transcripts that were downregulated (see Supplemental Tables S1, S2 and S3). We only selected transcripts lists that provided a fold ratio of the mean expression in hESCs to that in differentiated cells. Each list was mapped to Unigene build 176. When the mean value of expression in differentiated cells was zero, which occurred with ESTs and SAGE, the hESC/differentiation ratio was arbitrarily set at 50. Only genes with a fold ratio greater or equal to 2 (“hESC” genes), or lower or equal to 0.5 (“differentiation” genes) were selected. The Gene Ontology annotation analysis was carried out using the Fatigo+ tool on the Babelomics website (<http://babelomics.bioinfo.cipf.es>) using gene symbols [12]. Only annotation with a false discovery rates (FDR) adjusted P -value < 0.05 were considered significant.

Integrating Affymetrix GeneChip datasets obtained from distinct studies

In order to compare the transcriptome of hESCs to that of differentiated cell populations, we built an expression compendium by combining the U133A (Affymetrix, Santa Clara, USA) microarray data from 8 publications [13-20]. Indeed, we and others have shown that the GeneChip system (Affymetrix) allows direct comparison between datasets obtained in different centers, provided that the same chip and the same normalization are used [10, 21, 22]. The number of samples amounted to 217, including 24 hESC samples (11 different hESC lines). All samples were normalized before analysis with the GCOS 1.2 software (Affymetrix), using the “global scaling” method with a TGT value set to 100. The “detection call” can either be “*present*”, when the perfect match probes are significantly more hybridized than the mismatch probes, “*absent*” when both perfect match and mismatch probes display a

similar fluorescent signal, or “*marginal*” when the probeset does neither comply to the *present* nor to the *absent* call criteria. When several probesets measured the same gene, only the probeset with the maximal number of *present* detection call across all samples was selected. This step reduced the list of probesets to 14 074. This dataset is available as Supplemental Table S4 and can be accessed on our website <http://amazonia.montp.inserm.fr>.

Hierarchical clustering

Hierarchical clustering was carried out on the *detection call* data with the CLUSTER and TREEVIEW software packages [23]. The value 1 was assigned to *present* calls, -1 to *absent* calls and 0 to *marginal* calls. This matrix was clustered without further mathematical transformation. Only genes were clustered, *i.e.* the order of the samples is the order used on the *Amazonia!* website, and is based on grouping according to the embryonic germ layer origin of the sample.

hESC lines and karyotype

The list of hESC lines used and their respective karyotype are listed in Table 1.

hESC culture

After approval from the French Ministry of Research, the French Ministry of Health and the Agence de la Biomédecine, HUES1 and HUES3 hESCs were imported from Douglas Melton’s laboratory (Harvard University, MA, USA) and cultured as described [24]. Cells were passaged every 3-4 days enzymatically with 0.25% trypsin/EDTA (Invitrogen, Cergy Pontoise, France) and cultured in knockout Dulbecco’s modified essential medium (Invitrogen) without plasmanate, with 10% KO-SR (Invitrogen), 2 mM L-glutamine, 1x non-essential amino acids, 0.05 mM β -mercaptoethanol, 10 ng/ml FGF2 (Abcys, Paris, France). Medium was replaced daily. HUES1 and HUES3 were cultured on murine embryonic fibroblasts (MEF) obtained from E13 ICR mice embryos (Harlan, Gannat, France) or on human foreskin fibroblasts (hFF) in porcine skin gelatin coated (Sigma-Aldrich, St. Quentin

Fallavier, France) 6 wells dishes. The hFF cell lines SM1 and SM3 were derived from respectively 75 and 3 years old patients undergoing foreskin reduction. Informed consent was obtained from the patient or the patient's parents. MEF were cultured in DMEM with 10% fetal calf serum (FCS) and hFF in DMEM with 20% FCS. MEF and hFF were mitotically inactivated by mitomycin-C (2h at 10 µg/ml). These hESC expressed POU5F1, TRA-1-60, TRA-1-81, displayed phosphatase alkaline activity, and were able to differentiate into embryoid bodies that expressed differentiation markers of astrocytic lineage (GFAP) or endodermal lineage (alpha-feto protein) (see Supplemental Figure S1 and data not shown).

The HS293 and HS235 hESC lines were cultured in the Department of Obstetrics and Gynecology, (CLINTEC) at the Karolinska University Hospital as described [25]. Briefly, hESC were cultured on hFF (CRL-2429; American Type Culture Collection, Manassas, VA) mitotically inactivated by irradiation (40 Gy), in KO-DMEM 20% knockout SR, 2 mM Glutamax, 0.5% penicillin–streptomycin, 1% nonessential amino acids, 0.5 mM β-mercaptoethanol (all from Gibco Invitrogen Corporation, Paisley, Scotland), 1% insulin-transferrin-selenium (Sigma-Aldrich) and 8 ng/mL of bFGF (R&D Systems, Oxford, UK).

For karyotype analysis, hESC cells were treated with 75ng/ml final Karyomax Colcemid (Invitrogen) for 1h, trypsinized, incubated in 0.0375M KCl for 20 min, and fixed in fresh 3:1 methanol/acetic acid solution. 1 to 2 spreads were counted for chromosome number and 12 to 16 banding patterns were analyzed at 300–500 bands resolution.

Flow cytometry analysis

Human ES cells and fibroblasts were dissociated with trypsin (0.25%)-EDTA (1mM) (GIBCO) for 3 min. Cells were then washed with PBS and incubated for 30 min at 4°C in PBS with the corresponding monoclonal antibody (MAb): anti-CD24 MAb conjugated to phycoerythrin (PE) (dilution 1:50) (clone ALB9, Immunotech, Marseille, France) and/or anti-CD44 MAb conjugated to fluorescein isothiocyanate (FITC) (dilution 1:50) (clone J-173,

Immunotech). After PBS washes, cells were suspended in Facsflow (Becton Dickinson, San Jose, CA) and fluorescence was analyzed with a FACSCalibur flow cytometer (Becton Dickinson) or sorted with a FACSAria cell sorter (Becton Dickinson). Appropriate isotype controls were included in all analyses.

Immunofluorescence

hESCs cultured on coverslips were fixed for 20 min in 4% paraformaldehyde and washed three times in PBS. Cells were permeabilized with 0.1% Triton X-100. After blocking at room temperature for 60 min in PBS with 5% donkey serum (S30, Chemicon international, Temecula, CA), cells were incubated for 1 hour at room temperature with primary antibody diluted in PBS with 5% donkey serum: POU5F1/OCT3/4 (sc 9081, Santa Cruz Biotechnology, Santa Cruz, CA; 1:300) and SEMA6A (AF1146, R&D, Abington, United Kingdom; 1:50). Cells were washed three times in PBS and incubated for 1 hour at room temperature with Alexa Fluor® 488 Donkey anti-Rabbit (A-11034; Molecular Probes; 1:1000) and Cy3 Donkey anti-Goat (Jackson ImmunoResearch; USA; 1:400) secondary antibodies, for POU5F1 and SEMA6A respectively. Unbound antibodies were removed by three washes in PBS. Hoechst staining was added to the first wash (Sigma, 5 µg/ml).

Results

Compiling hESC expression profiles

As of October 1st, 2006, we identified 38 original studies, 1 protocol description and 4 reviews analyzing the transcriptome of hESCs (Table 2). The original studies used various hESCs, control cells and gene expression analysis techniques (summarized in Supplemental Table S1). 28 different hESC lines were used and transcriptome techniques included microarrays (7 different types of chips), ESTs scanning, SAGE, MPSS and Illumina beads. One study compared chromosome immuno-precipitation (ChIP) chip data with transcriptome data in hESCs [10]. Nevertheless, some common features emerged such as the frequent investigation of the H1, H9, and BG01 hESCs (in 12, 12 and 11 studies respectively), and the use of the GeneChip (Affymetrix) microarray system (in 16 studies) (see supplemental table S1).

Meta-analysis of genes differentially expressed between hESCs and non-pluripotent cells

One main objective of large scale gene expression analyses of hESCs is to identify the set of genes that are overexpressed in this unique cell type (“hESC” genes) or underexpressed (“differentiation” genes). We reasoned that bona fide “hESC” or “differentiation” genes would be repeatedly uncovered by independent groups, regardless of the hESC lines, the control cells, the assay format or the statistical method that had been used. We collected, from these 38 original studies, 20 lists of transcripts overexpressed in hESCs (“hESC genes”) and 11 lists of genes underexpressed in hESCs (“differentiation genes”).

The 20 lists of hESC genes comprised 5567 different genes. As illustrated in Figure 1A, we observed a marked heterogeneity between these lists, with only 1076 genes found overexpressed in hESCs by three or more independent studies, 48 genes by 10 or more studies and only 1 gene by all 20 lists (see Supplemental Table S2). The 48 genes found overexpressed in at least 10 studies are listed in Table 3. Of note, the pivotal ES transcription

factor POU5F1/OCT3/4 is the one gene found by all 20 lists, whereas genes found in at least 10 studies include the transcription factors *NANOG* and *SOX2*, and the growth factors *TDGF1/CRIPTO* and *Galanin (GAL)* that are known to be highly expressed by hESCs. Thus, according to hESC transcriptome analyses published to date, this list of 1076 genes found overexpressed in hESC cells by three or more studies can be viewed as a “consensus hESC gene list”.

In order to get further insights into this hESC list, we built an expression compendium by combining the data from 5 publications using the U133A GeneChip microarray to analyze hESC transcriptome and 3 publications providing the transcriptome of various normal fetal and adult tissues (see material and method and Supplemental Table S4). This compendium included the gene profiling of 24 hESC samples and more than 190 various fetal and adult tissues samples. A heat map was generated for the “consensus hESC gene list” in this expression compendium based on the *detection call* provided by the GCOS 1.2 software. The *detection call* is a way to evaluate whether a gene is expressed or not in a given sample. Hierarchical clustering (Figure 1C) delineated four major clusters of genes: cluster (a) was a group of 40 genes specifically detected in hESCs (“hESC specific” genes, Table 4), including expected genes such as *POU5F1/OCT3/4*, *NANOG*, *TDGF1*, *LIN28*, *CLDN6*, *GDF3*, *DNMT3A*, but also genes such as *CYP26A1*, *HELLS* or *GPR19*, cluster (b) featured genes that were detected in both hESCs and CNS samples such as the GABA receptors *GABRB3* and *GABRA5*, and the growth factor *FGF13*, cluster (c) genes detected in samples characterized by a high mitotic index such as *SKP2*, *MYC*, the cyclin *CCNA2* and the MCM genes *MCM2*, *MCM5*, *MCM6* and *MCM7*, cluster (d) genes overexpressed in hESCs but also expressed in a majority of the tissues included in this dataset such as *PGK1*, *HSPA9B*, and the ribosomal genes *RPLP0*, *RPL6*, *RPL7* and *RPL24*. The complete lists of genes composing these clusters are available as Supplemental Table S5. The expression histograms of the 40 hESC specific

genes are shown in Supplemental Figure S2. These results show that, though all 1076 genes of the “consensus hESC gene list” have been found overexpressed in hESCs compared to non-hESC samples by at least three different studies, 40 genes are indeed hESC specific (cluster a) but most are nonetheless also expressed in adult tissues to various extent.

The 11 lists of “differentiation” genes summed up to 4798 genes and we noted a similar heterogeneity as in the “hESC” lists (Supplemental Table S3). Out of these 4798 genes, only 783 were found underexpressed in hESCs by at least 3 different studies, composing a “consensus differentiation gene list”, three genes, lumican, collagen 1A1 and 3A1, by nine studies and none by all 11 studies (Figure 1B). Table 5 shows 30 selected genes found by at least 6 studies, which include the *bone morphogenetic proteins (BMP) 1* and *4*, the *keratins 7* and *18*, *insulin-like growth factor 2 (IGF2)*, *Heart and neural crest derivatives expressed 1 (HAND1)* and the transducing chain *IL6ST/GP130*. The complete “consensus differentiation gene list” can be found in Supplemental Table S3.

We compared functional Gene Ontology (GO) annotations of the hESC genes to the differentiation genes. Several functional annotations were more represented in each category (Figure 1D). There were significantly more genes involved in “metabolism”, “mitosis”, “RNA splicing”, “nuclear pore”, “DNA repair” in the hESC gene list, reflecting the intense proliferation, DNA replication and DNA remodeling taking place in these cells. Conversely, GO annotations such as “organ development”, “skeletal development”, “extracellular matrix”, “cell adhesion”, “cell communication”, “integral to plasma membrane”, “signal transduction”, were significantly more frequent among genes upregulated in differentiated tissues, in agreement with the idea that hESC differentiation mimics early organogenesis, which is associated with the development of complex cell-cell communication and cell-extra cellular matrix (ECM) interactions.

CD24 and SEMA6A are hESC markers

We next inquired whether we could find, among the hESC genes, new markers that may be useful to identify, isolate and qualify hESCs *in vitro*. We focused on two cell surface hESC genes: *CD24* and *SEMA6A*. These two genes have been found to be overexpressed in hESCs by respectively nine and fifteen studies, and are therefore good candidates as new hESC markers.

CD24 is a sialoglycoprotein known to be expressed on mature granulocytes and B cells subpopulations [26]. In addition to these hematopoietic cell types, microarray data from the expression compendium evidenced a high CD24 mRNA expression in keratinocytes, pancreas and thyroid, whereas most neural tissues, muscle, liver and testis did not express CD24 (Figure 2A). Upon differentiation of hESCs into embryoid bodies (EBs), CD24 expression is markedly downregulated (Figure 2B). Importantly, human foreskin fibroblasts (hFF) samples did not express CD24 (Figure 2C, 2F). Hence, we investigated whether CD24 could discriminate hESCs from fibroblasts in culture. We analyzed by flow cytometry the hESC lines HUES1, HUES3, HS293 and HS235 [24, 25] cultured on hFF and evidenced two distinct cell populations, one CD24⁺ and one CD24⁻ (Figure 2E). To demonstrate that the CD24⁺ population corresponded to hESCs and the CD24⁻ population to hFF, we took advantage of CD44, a strong fibroblast marker not expressed by hESCs (Figures 2D, 2G). Double staining of hESCs cultured on hFF showed that these two markers were expressed in a mutually exclusive manner and delimited a hESC CD24⁺CD44⁻ population that did not overlap with the fibroblast CD24⁻CD44⁺ population (Figure 2H). Using CD24/CD44 labeling, we were able to separate hESC from fibroblasts and obtain pure hESC populations that recovered the cell sorting procedure and grew *in vitro* while retaining POU5F1 and cardinal cell surface markers of pluripotency expression (Supplemental Figure S3 and data not shown).

SEMA6A is a class 6 semaphorin [27], *i.e.* transmembrane with cytoplasmic domain, known to be expressed in developing neural tissue. We recently reported the expression of this semaphorin in cumulus oophorus cells [28]. Comparison of RNA expression in hESCs and normal adult tissues showed that in addition to hESCs, SEMA6A was also expressed at high level in adult samples from the central and peripheral nervous system and placenta (Figure 2I). As for CD24, SEMA6A mRNA is downregulated upon EB differentiation and is not detected in human fibroblasts (Figure 2J and K). Immunofluorescence analysis showed a membrane localization of SEMA6A on hESCs, in contrast to POU5F1/OCT3/4 which had a strict nuclear localization (Figure 2 L-M). In summary, we showed that CD24 and SEMA6A have indeed a preferential expression in hESCs, that this expression is confirmed at the protein level, and is declining upon EB differentiation. Thus these markers can be used to discriminate hESCs from feeder fibroblasts

hESC transcriptome data visualization through an open access web interface

We developed a website, *Amazonia!* (<http://amazonia.montp.inserm.fr>), to allow the scientific community access to these public data. This website is dedicated to the visualization of large, publicly available, human transcriptome data (Le Carrouer et al., manuscript submitted). A main topic of this website is human embryonic stem cells. Data are visualized as expression histograms with a color code facilitating the recognition of cell type. Genes are accessed either by key words or through lists of genes. Most interestingly, when data were obtained using the same platform format, sample labeling and data normalization, it was possible to combine different experiments in one single virtual experiment. The U133A expression compendium comprises for example more than 200 different samples from 8 publications including hESCs and normal adult tissues. Thus, *Amazonia!* provides the expression profile of about 15 000 different genes in about 100 different tissues types or purified cell populations including 11 hESCs (H1, H9, HS181, HS235, HS237, FES21, FES22, FES29, FES30, J6,

HES-2). Figure 3A illustrates this feature of our website with the expression histograms of five hESC specific genes (*POU5F1*, *NANOG*, *GPR19*, *Helicase lymphoid-specific (HELLS)* and the *cytochrome P450 CYP26A1*), two genes expressed in nonlineage-differentiated cells (*HAND1* and *IGF2*), two factors highly expressed by human fibroblasts and smooth muscle that may contribute to the supporting properties of fibroblast to hESC culture (*Gremlin (GREM1)* and *Matrix metalloproteinase 1 (MMP1)*), one hematopoietic marker (*CD45*), one central nervous system marker (*Glial fibrillary acidic protein (GFAP)*) and one ubiquitously expressed gene (*Ribosomal protein L3 (RPL3)*).

Another important feature of *Amazonia!* is the possibility to compare on the same web page the expression of a gene of interest in various datasets. Figure 3B shows that the expression of *Frizzled 7 (FZD7)* was markedly upregulated in hESCs, was downregulated during non-lineage differentiation into EBs, and was also highly expressed in embryonal carcinoma and Yolk sac carcinoma samples. The combination of these three histograms evidences a preferential *FZD7* mRNA expression in normal and malignant embryonic cells, suggesting that *FZD7* may play a major role in these pluripotent cell types.

In order to facilitate access for the scientific community to the lists of genes from published transcriptome analyses, we implemented a list manager in our *Amazonia!* website. Thus, one can access these lists straightforwardly and obtain an expression histogram for each gene in various public transcriptome collections. This feature is of particular interest to challenge a list of genes, for example a “hESC” gene list, with other hESC datasets or even with non-hESC datasets such as cancer datasets. Indeed, once a gene is selected, the user can navigate between various *thematic pages*, switching for instance from the “stem cells” page to the “leukemia and lymphoma”, “lung cancer” or other pages. Using this feature of the *Amazonia!* website, we could observe that the sialoglycoprotein *CD24* was also highly expressed in acute

lymphoblastic leukemia, lung cancer and glioma samples when compared to the corresponding normal samples (Figure 3C and data not shown).

Discussion

Human ESCs are remarkable by their ability both to self renew and to generate virtually any kind of cell type, hence carrying many hopes for cell therapy. It is anticipated that genome wide expression analyses, by providing an extensive molecular taxonomy, will help understanding this unique cell model of stem cell pluripotency. Transcriptome results can be viewed as a non-biased, genome wide, expression catalog. Many groups have published the transcriptome of hESCs, but, as a direct consequence of the massive data generated, access to this data on a routine basis was precluded for most researchers. Therefore, the construction of a database collecting publicly available hESC transcriptome and accessible through a user friendly interface is of utmost interest for the hECS researchers' community. We found 38 original studies or reviews analyzing the transcriptome of hESCs. Expression data and gene lists extracted from these studies were included into *Amazonia!* and are now readily accessible. Interestingly, the frequent use of the U133A oligonucleotide microarrays allowed us to construct a virtual expression dataset of about 15 000 different genes in more than 200 tissue samples from various origins, including 24 hESC samples. Hence, the expression of each gene in hESCs is directly contrasted to that of normal fetal and adult tissues, as illustrated in Figure 3A.

Unearthing the hESC gene panoply may help to define what makes hESCs unique. To achieve this goal, most studies compared hESC transcriptome data to that of more differentiated cell types and obtained lists of genes over- or underexpressed in hESCs. Comparison of different transcriptome surveys of hESCs gave us the unique opportunity to identify genes that were identified by several authors as differentially expressed in hESCs. However, a striking heterogeneity was observed between the 20 lists of hESC genes, and only 48 genes were

found to be highly enriched in hESCs in at least ten or publications, among 5567 genes found in at least one. Some of these differences may be explained by platform-to-platform or lab-to-lab variability, but this is likely not the main explanation as suggested by transcriptome platform comparisons [22]. Rather, the differences between the hESC cells lines used, the control samples, the specific caveats of each transcriptome analysis technique and the statistical methodologies likely contribute to these disparities (see Supplemental Table S2). For instance, the homeobox transcription factor *NANOG*, which is universally expressed in hESC, was not reported by Sperger et al. because no probe for *NANOG* was present on their microarray [29], nor was it reported by Brandenberger et al. because the differential regulation of *NANOG* in their in vitro differentiation model did not meet their stringent statistical criteria [30]. *ZFP42* (the human homolog of murine *rex1*) was never listed by MPSS studies because its MPSS signature has repeat sequences [31]. Another pitfall impacting on differentially expressed genes lists comparisons and contributing to the “small intersection” problem [32] is that in order to be at the intersection of 20 lists, a gene must have fulfilled 20 times the statistical filter, which it does with a probability equal to the product of the probabilities of each test. A way to circumvent this difficulty would be to obtain the raw data from these studies and to apply specific statistical tests [33]. However, the raw data were not available for many studies analyzed here, which prevented us from applying this approach in our study. Nevertheless, the 1076 hESC genes list provides the opportunity to the scientific community to examine the genes that have found over- or underexpressed in hESC by several authors. These lists provide further molecular insight into the biology of this unique stem cell model and are now starting points for many new research directions in the field of hESC. In the future, it will be interesting to extend this list by investigating additional hESC lines. The collection of additional transcriptome data is

ongoing and we will update the database according to new publications on the hESC genome expression.

As one can easily notice by browsing the “consensus hESC gene list” in the U133A “embryonic and adult samples” dataset, we found very few genes that are completely hESC specific. Indeed, most genes found overexpressed in hESCs are also expressed in other tissues (Figure 1C), and only 40 genes, grouped in cluster (a), comprised genes which expression was not detected in most other tissues (see supplemental Figure S2). Of note, genes can only be labeled as hESC “specific” in respect of the different tissues and cell populations that have been tested. As new cell types will be investigated, some of these genes may be found expressed in these new samples and thereby cease to be specific to hESCs. Specificity also depends on the sensitivity of the assay investigating expression of a given gene. For example, *POU5F1/OCT3/4* has been reported to be expressed in germinal cells and even in bone marrow by RT-PCR assays [34, 35]. By contrast, microarray analysis show that this gene, clearly expressed at a high level in hESCs and teratocarcinoma samples, is neither detected by this technique in testis, ovary, bone marrow samples, nor in a pure oocyte population (Figure 3A and unpublished data). These observations suggest that the properties of hESCs, comprising self-renewal, unrestrained proliferation and pluripotency, are mediated by the expression of few specific genes, if any, together with genes which individually are not hESC specific, but whose combined expression is specific to hESCs. The “consensus hESC gene list” should encompass many of those genes contributing to the embryonic stem cell characteristics.

Transcriptome approaches have several limitations. Each technique has its own technical limits, but this meta-analysis partly circumvented these by the simultaneous analysis of different, complementary methods. Another drawback is that by looking at the transcriptome, we consider gene expression at the RNA level only, eluding all forms of post-transcriptional

regulation. It will therefore be important in the future to look the differential expression of the genes described in the hESC and differentiation lists at these hESC and differentiation gene lists at the protein level. For that matter, this meta-analysis provides a list of pertinent genes for further protein validation. In line with this proposition, we chose to investigate more thoroughly two cell surface protein-encoding genes, which may serve as new hESC markers: *CD24* and *SEMA6A*. Though these two genes have been found to be overexpressed in hESCs by 9 and 15 studies, respectively, we found that hESCs shared this RNA expression with several adult tissues as substantiated by microarray results (Figure 2). However, purification of hESCs requires only distinguishing them from differentiating stem cells and from the co-cultured feeder cells. *CD24* expression is low in differentiating hESCs, and is absent in human fibroblasts. We were thus able to purify by flow cytometry pure hESC populations. This provides a new tool to isolate highly enriched populations of hESCs for subsequent experiments, including microarray analysis. We observed that *CD24* was also highly expressed on various malignant cell types, as previously reported [36, 37]. Since *CD24* is a ligand for P-selectin, it has been suggested that *CD24* could be important in the dissemination of tumor cells by facilitating the interaction with endothelial cells [38]. The role of *CD24* in hESCs is less clear because P-selectin is not expressed by human fibroblasts nor by hESCs themselves (data not shown), suggesting that *CD24* may have other molecular functions. Semaphorins have been initially identified for their role in neuronal guidance as chemorepellents, but it is becoming clear that this large family of genes also plays important roles in organogenesis, vascularization, angiogenesis and B lymphocyte signaling. We show here a clear protein expression of semaphorin 6A in hESCs, with an overexpression in hESCs compared to many other cells types, suggesting a functional role for this transmembrane molecule in cell-to-cell interaction or signaling in hESCs.

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The clustering of the “consensus hESC gene list” based on the “detection call” identified a cluster of 40 genes with a high specificity in hESCs, with no expression in most samples from more than 100 different fetal and adult cell tissues (Table 4). In addition to genes clearly expected such as *POU5F1/OCT3/4*, *NANOG* or *TDGF1*, this cluster included genes whose hESC specificity had been overlooked. For example the expression of the G protein-coupled receptor 19 (GPR19) is restricted to hESCs and, if functional, may offer a possibility for *in vitro* intervention on proliferation or pluripotency of hESCs. Other hESC specific genes comprise the cytochrome *P450 CYP26A1* which is responsible for retinoic acid degradation [39] or the helicase *HELLS* which is expressed at a moderate level in a few lymphoid samples but most importantly in hESCs and could be involved in DNA strand separation, including replication, repair, recombination, and transcription [40] (Figure 3A). Our meta-analysis also spotted additional interesting genes such as *FZD7* which was identified as the frizzled receptor preferentially expressed on hESCs. Based on this expression, we hypothesize that *FZD7* could be a major WNT receptor in hESCs. Thus, *FZD7* could contribute to the pluripotency signal mediated by WNT previously reported in hESCs [41]. Regarding the “consensus differentiation gene list”, many genes are clearly related to differentiation such as collagen and keratin genes. We note that the transducing chain *IL6ST/GP130*, which is necessary to convey the signals from the IL-6 growth factor family including LIF, is expressed at a very low level in hESCs as compared to most differentiated adult tissues. This is in line with the largely accepted view that LIF signaling is dispensable for pluripotency in hESCs [2].

Conclusion

We analyzed 38 publications studying the hESC transcriptome. We propose a “consensus hESC gene list” and a “consensus differentiation gene list” that identifies the genes found

respectively up- or downregulated in hESCs compared to differentiated samples by at least three publications. We provide the first tool to directly visualize the expression of most human genes in hESCs, and provide direct comparison with their expression in many normal and malignant tissues. This tool may be considered as the first hESC expression Atlas online. By providing an easy access to this large public data, we hope that *Amazonia!* will help boosting the translation of these invaluable expression information into biological applications.

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Tables

Table 1 – hESC: laboratory of origin and karyotype

hESC	Laboratory of origin	Karyotype
HUES1	Douglas Melton, Harvard	p20 - 46,XX p30 - 46,XX p40 - 46,XX, t(1;10)
HUES3	Douglas Melton, Harvard	p23 - 46,XY p49 - 47,XXY
HS293	Outi Hovatta, Karolinska Institute	p49 - 46,XY
HS235	Outi Hovatta, Karolinska Institute	p75 - 46,XX

Table 2 – 43 original studies or reviews analyzing hESC transcriptome

Reference	Technique/Review
(Abeyta et al., 2004) [43]	Microarray
(Aghajanova et al., 2006) [44]	Microarray
(Armstrong et al., 2006) [45]	Microarray
(Beqqali et al. 2006)[46]	Microarray
(Bhattacharya et al., 2004) [47]	Microarray
(Bhattacharya et al., 2005) [48]	Microarray
(Boyer et al., 2005) [10]	ChIP chip
(Brandenberger et al., 2004b) [30]	ESTs
(Brandenberger et al., 2004a) [49]	MPSS
(Brimble et al., 2004) [50]	MPSS
(Brimble et al. 2006) [51]	Microarray
(Cai et al., 2005) [52]	Illumina BeadChips
(Calhoun et al., 2004) [53]	Microarray
(Darr et al., 2006) [54]	Microarray
(Dvash et al., 2004) [55]	Microarray
(Enver et al., 2005) [56]	Microarray
(Ginis et al., 2004) [57]	Microarray
(Golan-Mashiach et al., 2004) [14]	Microarray
(Hirst et al.,2006) [19]	Microarray
(Josephson et al. 2006) [58]	Illumina BeadChips
(Kim et al., 2006) [59]	Microarray
(Li et al. 2006) [60]	Illumina BeadChips
(Liu et al. 2006) [61]	Illumina BeadChips
(Miura et al., 2004) [62]	MPSS and EST
(Perez-Iratxeta et al., 2005) [20]	Microarray
(Player et al., 2006) [63]	Microarray
(Pyle et al., 2004) [64]	Review
(Rao M., 2004) [65]	Review
(Rao R. et al., 2004) [66]	Microarray
(Rao R. and Stice, 2004) [67]	Review
(Richards et al., 2004) [68]	SAGE

(Richards et al., 2006) [69]	SAGE
(Robson, 2004) [70]	Review
(Sato et al., 2003) [15]	Microarray
(Sato and Brivanlou, 2006) [71]	Microarray (protocol description)
(Skottman et al., 2005) [16]	Microarray
(Skottman et al., 2006) [72]	Microarray
(Sperger et al., 2003) [29]	Microarray
(Suarez-Fariñas et al., 2005) [33]	Meta-analysis
(Sun et al., 2006) [73]	Microarray
(Wei et al., 2005) [31]	MPSS
(Xu et al. 2002) [74]	Microarray
(Zeng et al., 2004) [75]	Microarray

Table 3 - 48 genes overexpressed in hESCs compared to differentiated cell types in at least 10 studies

Abbreviation	Name	Chr. (€)	Unigene	Nb of studies	Ratio Und/Dif Signal (€)
POU5F1	<i>POU domain, class 5, transcription factor 1</i>	6p21.31	Hs.249184	20	56.4
TDGF1	<i>Teratocarcinoma-derived growth factor 1</i>	3p21.31	Hs.385870	17	55.0
DPPA4	<i>Developmental pluripotency associated 4</i>	3q13.13	Hs.317659	16	30.1
LIN28	<i>Lin-28 homolog (C. elegans)</i>	1p36.11	Hs.86154	16	24.8
NANOG	<i>Nanog homeobox</i>	12p13.31	Hs.329296	15	88.9
DNMT3B	<i>DNA (cytosine-5-)-methyltransferase 3 beta</i>	20q11.2	Hs.251673	15	27.8
TERF1	<i>Telomeric repeat binding factor (NIMA-interacting) 1</i>	8q13	Hs.442707	15	18.9
SEMA6A	<i>Semaphorin 6A</i>	5q23.1	Hs.156967	15	12.3
M6PR	<i>Mannose-6-phosphate receptor (cation dependent)</i>	12p13	Hs.134084	15	10.6
SNRPN	<i>Small nuclear ribonucleoprotein polypeptide N</i>	15q11.2	Hs.525700	15	7.3
FLJ10884	<i>Hypothetical protein FLJ10884</i>	1p31.3	Hs.562195	14	260.7
LEFTY1	<i>Left-right determination factor 1</i>	1q42.1	Hs.278239	14	34.1
GAL	<i>Galanin</i>	11q13.2	Hs.278959	14	21.4
SEPHS1	<i>Selenophosphate synthetase 1</i>	10p14	Hs.124027	14	6.3
GABRB3	<i>Gamma-aminobutyric acid (GABA) A receptor, beta 3</i>	15q11.2-q1	Hs.302352	13	15.3
SOX2	<i>SR Y (sex determining region Y)-box 2</i>	3q26.3-q27	Hs.518438	13	15.3
LECT1	<i>Leukocyte cell derived chemotaxin 1</i>	13q14-q21	Hs.421391	12	37.2
LOC90806	<i>Similar to RIKEN cDNA 2610307I21</i>	1q32.3	Hs.157078	12	14.6
BUB1	<i>BUB1 budding uninhibited by benzimidazoles 1 homolog</i>	2q14	Hs.469649	12	11.2
PSIP1	<i>PC4 and SFRS1 interacting protein 1</i>	9p22.3	Hs.493516	12	5.4
INDO	<i>Indoleamine-pyrrole 2,3 dioxygenase</i>	8p12-p11	Hs.840	11	34.4
HELLS	<i>Helicase, lymphoid-specific</i>	10q24.2	Hs.546260	11	19.3
GPC4	<i>Glypican 4</i>	Xq26.1	Hs.58367	11	15.4
ITGB1BP3	<i>Integrin beta 1 binding protein 3</i>	19p13.3	Hs.135458	11	15.3
CYP26A1	<i>Cytochrome P450, family 26, subfamily A, polypeptide 1</i>	10q23-q24	Hs.150595	11	14.2
MCM5	<i>MCM5 minichromosome maintenance deficient 5</i>	22q13.1	Hs.517582	11	11.9
MTHFD1	<i>Methylenetetrahydrofolate dehydrogenase 1</i>	14q24	Hs.435974	11	8.7
PPAT	<i>Phosphoribosyl pyrophosphate amidotransferase</i>	4q12	Hs.331420	11	8.3
SLC16A1	<i>AKR7 family pseudogene</i>	1p12	Hs.75231	11	7.8
NASP	<i>Nuclear autoantigenic sperm protein (histone-binding)</i>	1p34.1	Hs.319334	11	7.4
DLG7	<i>Discs, large homolog 7 (Drosophila)</i>	14q22.3	Hs.77695	11	6.7
UGP2	<i>UDP-glucose pyrophosphorylase 2</i>	2p14-p13	Hs.516217	11	6.1
MSH2	<i>MutS homolog 2, colon cancer, nonpolyposis type 1</i>	2p22-p21	Hs.156519	11	3.4
ZIC3	<i>Zic family member 3 heterotaxy 1</i>	Xq26.2	Hs.111227	10	30.7
PIM2	<i>Pim-2 oncogene</i>	Xp11.23	Hs.496096	10	18.5
HRASLS3	<i>HRAS-like suppressor 3</i>	11q12.3-q1	Hs.502775	10	16.8
ALPL	<i>Alkaline phosphatase, liver/bone/kidney</i>	1p36.1-p34	Hs.75431	10	15.0
AASS	<i>Amino adipate-semialdehyde synthase</i>	7q31.3	Hs.433075	10	13.8
MGST1	<i>Microsomal glutathione S-transferase 1</i>	12p12.3-p1	Hs.389700	10	10.9
GDF3	<i>Growth differentiation factor 3</i>	12p13.1	Hs.86232	10	9.5
HSPA4	<i>Heat shock 70kDa protein 4</i>	5q31.1-q31	Hs.90093	10	9.2
PHF17	<i>PHD finger protein 17</i>	4q26-q27	Hs.12420	10	9.2
UNG	<i>Uracil-DNA glycosylase</i>	12q23-q24.	Hs.191334	10	6.7
USP9X	<i>Ubiquitin specific peptidase 9, X-linked</i>	Xp11.4	Hs.77578	10	5.7
CEBPZ	<i>CCAAT/enhancer binding protein zeta</i>	2p22.2	Hs.135406	10	5.4
CRMP1	<i>Collapsin response mediator protein 1</i>	4p16.1-p15	Hs.135270	10	5.3
BMPRI1A	<i>Bone morphogenetic protein receptor, type IA</i>	10q22.3	Hs.524477	10	5.1

(£) Chromosomal location. (§) Ratio of mean expression in hESC samples to mean expression in non-hESC samples.

Table 4 - 40 genes specifically expressed in hESCs (cluster “a” in Figure 1C)

Abbreviation	Name	Chr. (£)	Unigene	Nb of studies	Ratio Und/Dif Signal (§)	Presence Call in non-hESC tissues (#)
NANOG	Nanog homeobox	12p13.31	Hs.329296	15	88.9	0
POU5F1	POU domain, class 5, transcription factor 1	6p21.31	Hs.249184	20	56.4	0
LIN28	lin-28 homolog (C. elegans)	1p36.11	Hs.86154	16	24.8	0
CDC25A	cell division cycle 25A	3p21	Hs.1634	5	19.4	0
PRDM14	PR domain containing 14	8p21-p12	Hs.287532	6	13.5	0
SLD5	SLD5 homolog	8p11.21	Hs.521557	5	13.2	0
C14orf115	chromosome 14 open reading frame 115	14q24.3	Hs.196530	9	12.9	0
GDF3	growth differentiation factor 3	12p13.1	Hs.86232	10	9.5	0
GPR19	G protein-coupled receptor 19	12p12.3	Hs.92458	6	6.3	0
GPR23	G protein-coupled receptor 23	Xq13-q21.1	Hs.522701	3	2.7	0
FLJ10884	hypothetical protein FLJ10884	1p31.3	Hs.562195	14	260.7	1
ZIC3	Zic family member 3 heterotaxy 1	Xq26.2	Hs.111227	10	30.7	1
CLDN6	claudin 6	16p13.3	Hs.533779	9	14.4	1
CYP26A1	cytochrome P450, family 26, A1	10q23-q24	Hs.150595	11	14.2	1
CXorf15	chromosome X open reading frame 15	Xp22.2	Hs.555961	3	12.4	1
HESX1	homeo box (expressed in ES cells) 1	3p21.2-p21	Hs.171980	5	10.3	1
KIAA0523	KIAA0523 protein	17p13.2	Hs.370166	4	6.5	1
MGC3101	hypothetical protein MGC3101	16q24.3	Hs.301394	5	5.0	1
CHEK2	CHK2 checkpoint homolog (S. pombe)	22q11	Hs.558896	7	3.3	1
RNU3IP2	RNA, U3 small nucleolar interacting protein 2	3p21.2	Hs.153768	4	3.0	1
GJA7	gap junction protein, alpha 7	17q21.31	Hs.532593	4	2.3	1
ETV4	ets variant gene 4	17q21	Hs.434059	9	9.8	2
DNMT3A	DNA (cytosine-5)-methyltransferase 3 alpha	2p23	Hs.515840	5	4.2	2
DCC1	defective in sister chromatid cohesion homolog 1	8q24.12	Hs.315167	5	3.5	2
SLC5A6	solute carrier family 5, member 6	2p23	Hs.435735	3	2.9	2
TDGF1	teratocarcinoma-derived growth factor 1	3p21.31	Hs.385870	17	55.0	3
PWP2H	PWP2 periodic tryptophan protein homolog	21q22.3	Hs.449076	4	27.0	3
ABHD9	abhydrolase domain containing 9	19p13.12	Hs.156457	3	9.7	3
EPHA1	EPH receptor A1	7q34	Hs.89839	7	8.0	3
MYBL2	v-myb homolog-like 2	20q13.1	Hs.179718	3	4.0	3
PRO1853	hypothetical protein PRO1853	2p22.2	Hs.433466	5	5.9	4
ORC1L	origin recognition complex, subunit 1-like (yeast)	1p32	Hs.17908	10	3.9	4
MCM10	minichromosome maintenance deficient 10	10p13	Hs.198363	5	3.8	4
FLJ20105	FLJ20105 protein	Xq13.1	Hs.47558	5	3.3	4
ORC2L	origin recognition complex, subunit 2-like (yeast)	2q33	Hs.444870	3	2.6	4
FLJ12484	interferon stimulated exonuclease like 1	15q26.1	Hs.37747	6	4.0	5
HELLS	helicase, lymphoid-specific	10q24.2	Hs.546260	11	19.3	6
BRRN1	barren homolog (Drosophila)	2q11.2	Hs.308045	5	4.6	6
RBM14	RNA binding motif protein 14	11q13.2	Hs.11170	3	4.1	7
DTYMK	deoxythymidylate kinase (thymidylate kinase)	2q37.3	Hs.471873	3	3.1	7

(£) Chromosomal location. (§) Ratio of mean expression in hESC samples to mean expression in non-hESC samples. (#) number of non-hESC samples with a detection call “Present” (out of 193 non-hESC samples).

Table 5 – 30 selected genes underexpressed in hESCs compared to differentiated cell types and found by at least 6 studies

Abbreviation	Name	Unigene	Chr. (£)	Nb of studies	Ratio Dif/Und Signal (§)
LUM	Lumican	Hs.406475	12q21.3	9	18.1
COL3A1	Collagen, type III, alpha 1	Hs.443625	2q31	9	14.0
COL1A1	Collagen, type I, alpha 1	Hs.172928	17q21.3	9	5.8
IGFBP3	Insulin-like growth factor binding protein 3	Hs.450230	7p13-p12	8	14.1
ACTA2	Actin, alpha 2, smooth muscle, aorta	Hs.500483	10q23.3	8	14.0
P4HA2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase A2	Hs.519568	5q31	8	5.5
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Hs.111779	5q31.3	8	4.7
COL1A2	Collagen, type I, alpha 2	Hs.489142	7q22.1	8	4.6
KRT18	Keratin 18	Hs.406013	12q13	8	3.8
DCN	Decorin	Hs.156316	12q13.2	7	39.9
BMP4	Bone morphogenetic protein 4	Hs.68879	14q22	7	15.0
COL5A1	Collagen, type V, alpha 1	Hs.210283	9q34	7	12.6
COL2A1	Collagen, type II, alpha 1	Hs.408182	12q13	7	10.5
RHOBTB3	Rho-related BTB domain containing 3	Hs.445030	5q15	7	6.4
NDRG1	N-myc downstream regulated gene 1	Hs.372914	8q24.3	7	6.1
COL6A3	Collagen, type VI, alpha 3	Hs.233240	2q37	7	6.0
CST3	Cystatin C	Hs.304682	20p11.21	7	5.3
KRT8	Keratin 8	Hs.533782	12q13	7	5.2
CD47	CD47 antigen (Rh-related antigen)	Hs.446414	3q13.1	7	3.5
HAND1	Heart and neural crest derivatives expressed 1	Hs.152531	5q33	6	61.9
IGF2	Insulin-like growth factor 2 (somatomedin A)	Hs.373908	11p15.5	6	23.6
CXCL14	Chemokine (C-X-C motif) ligand 14	Hs.483444	5q31	6	10.5
COL11A1	Collagen, type XI, alpha 1	Hs.523446	1p21	6	8.7
IGFBP7	Insulin-like growth factor binding protein 7	Hs.479808	4q12	6	8.0
IL6ST	Interleukin 6 signal transducer (gp130)	Hs.532082	5q11	6	7.6
COL5A2	Collagen, type V, alpha 2	Hs.445827	2q14-q32	6	6.8
KRT7	Keratin 7	Hs.411501	12q12	6	6.5
KRT19	Keratin 19	Hs.514167	17q21.2	6	6.5
BMP1	Bone morphogenetic protein 1	Hs.1274	8p21	6	6.1
CDKN1A/P21	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Hs.370771	6p21.2	6	4.6

(£) Chromosomal location. (§) Ratio of mean expression in non-hESC samples to mean expression in hESC samples.

Supplemental Tables:

- Supplemental Table S1 : details on the 43 hESC transcriptome publications including the hESC used, the number of genes found up and down regulated (Excel file)
- Supplemental Table S2 : 1959 genes found in at least two hESC lists, including the hESC consensus list (Excel file)
- Supplemental Table S3: 1560 genes found in at least two differentiation gene lists, including the consensus differentiation list (Excel file)
- Supplemental Table S4: signal and detection call of 14 074 probesets from the U33A microarray (Affymetrix) in 217 hESC and non-hESC samples from 8 studies (Excel file)
- Supplemental Table S5: 4 clusters of genes highlighted in figure 1C (Excel file)

Legends to Figures

Figure 1: Meta-analysis of published “hESC” and “differentiation” gene lists

1959 different genes were found overexpressed in hESCs compared to differentiated tissues by at least two out of 20 independent studies (purple pyramid) (A). Of these, 1076 genes were found by at least three studies, 48 genes by at least 10 studies and only 1 gene (POU5F1) by all studies. Eleven studies provided lists of genes downregulated in hESCs compared to more differentiated samples (yellow pyramid) (B). 1560 genes were found downregulated in hESC by at least two studies, 783 by at least 3 different studies, three by at least 9 different studies and none by all studies.

Heat map of gene expression detection for the hESC gene list across 24 hESC and 193 fetal and adult tissues samples analyzed with the U133A microarray (828 probesets) (C). Red stands for a “Present” detection call (i.e. gene expression confidently detected according to the GCOS 1.2 software), grey for “Marginal” and yellow for “Absent”. Cluster a: genes exclusively expressed in hESCs; b: gene expressed in hESCs and central nervous system samples; c: cell cycle genes; d: genes expressed in hESCs and in most tissues analyzed. The clustering was carried out using the Cluster and Treeview software. Only genes were clustered. hFF: human foreskin fibroblasts; OV: ovary; CNS: central nervous system; PNS: peripheral nervous system; SK: skin and keratinocytes; LU: lung; DT: digestive tract; TH: thyroid; AD: adipocytes; K&P: kidney and prostate; H&M: heart and muscle; HEMATO: various hematopoietic tissues; UT: uterus; PL: placenta.

Comparison of GO annotations between hESC genes and differentiation genes (D). We compared the frequency of GO annotations of genes overexpressed in hESCs to those underexpressed in hESCs. The statistical analysis was carried out using the Babelomics webtool (<http://babelomics.bioinfo.cipf.es/>). Histograms show the percentage of genes with

the specified GO annotation in the group of genes overexpressed in hESCs (purple) or in differentiated tissues (yellow). *P* : adjusted FDR *P*-value.

Figure 2: CD24 and SEMA6A are two new hESC markers

Expression histograms of *CD24* across the U133A compendium (A). These histograms were obtained from our *Amazonia!* website from the U133A oligonucleotide microarray (Affymetrix) datasets. The Y-axis features the signal value obtained with GCOS 1.2 software. Microarray expression histograms of *CD24* gene before and after non-lineage differentiation into EBs (data from the study of [56]) (B). Mean U133A microarray signal value of *CD24* (C) and *CD44* (D) in 24 hESC samples versus two human fibroblasts samples. Flow cytometry analysis of *CD24* on HUES1 hESCs co-cultured with hFF (E), of hFF alone (F) and of *CD44* on hFF alone (G), and double *CD24* and *CD44* labeling of HUES1 (passage 37), HUES3 (p43), HS235 (p30) and HS293 (p68) cells co-cultured with hFF (H). Expression histogram of *SEMA6A* (I). Microarray expression histograms of *SEMA6A* gene before and after differentiation into EBs (data from the study of [56]) (J). Mean U133A microarray signal value of *SEMA6A* in 24 hESC samples versus two human fibroblasts samples (K). Indirect immunofluorescence localization of *SEMA6A*, *POU5F1/OCT3/4*, or both and corresponding Hoechst nuclear staining in the HUES1 (L) and HUES3 hESCs (M) cultured on murine embryonic and human fibroblasts, respectively. Scale bar: 25µm. White arrow: MEF (L) or hFF (M).

Figure 3: expression of selected genes using Amazonia!

Expression histograms of five hESC specific genes (*POU5F1*, *NANOG*, *GPR19*, *HELLS* and *CYP26A1*), two genes expressed in nonlineage-differentiated hESC cells (*HAND1* and *IGF2*), two factors highly expressed by human fibroblasts and smooth muscle that may contribute to the supporting properties of fibroblasts to hESC culture (*Gremlin* and *MMP1*), one

hematopoietic marker (*CD45*), one central nervous system specific gene (*GFAP*) and one ubiquitously expressed gene (*Ribosomal protein L3 (RPL3)*)(A).

FZD7 expression in three distinct datasets using the *Amazonia!* web tool: in the “Embryonic and adult normal tissues” series (Affymetrix U133A microarray, from 8 different publications) (B), in the “hESC” series from Enver et al. (Affymetrix U133A) [56] (C) and in the “hESC, germinal and embryonic malignancies” series (Stanford microarray) [29]. EC: embryonic carcinoma; YSC: Yolk sac carcinoma. (D)

Comparison of the expression of *CD24* mRNA across normal (NL) and malignant lung (ML) samples (E), and across normal hematopoietic (NH) and B acute lymphoblastic leukemia (ALL) and T ALL samples (F).

Supplemental figures:

- Supplemental figure S1: HUES1 hESC express pluripotency markers and are able to form embryoid bodies expressing differentiation markers. Scale bar: 12.5 μ m
- Supplemental figure S2: expression histograms of 40 “hESC specific” genes in the U133A microarray (Affymetrix) dataset comprising hESC and non-hESC samples from 8 studies, using the *Amazonia!* Website (<http://amazonia.montp.inserm.fr>).
- Supplemental figure S3: flow cytometry CD24+CD44- purified HUES1 and HUES3 cells were cultured for two weeks after cell sorting and assessed for POU5F1/OCT3/4 expression by immnofluorescence.